Trimethylamine *N*-oxide metabolism by abundant marine heterotrophic bacteria

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Abstract

Trimethylamine *N*-oxide (TMAO) is a common osmolyte found in a variety of marine biota and has been detected at nanomolar concentrations in oceanic surface waters. TMAO can serve as an important nutrient for ecologically important marine heterotrophic bacteria, particularly the SAR11 clade and marine *Roseobacter* clade (MRC). However, the enzymes responsible for TMAO catabolism and the membrane transporter required for TMAO uptake into microbial cells have yet to be identified. We show here that the enzyme, TMAO demethylase (Tdm), catalyses the first step in TMAO degradation. This enzyme represents a large group of proteins with an uncharacterized domain (DUF1989). The function of TMAO demethylase in a representative from the SAR11 clade (strain HIMB59) and in a representative of the MRC (*Ruegeria pomeroyi* DSS-3) was confirmed by heterologous expression of *tdm* (the gene encoding Tdm) in *Escherichia coli*. In *Ruegeria pomeroyi*, mutagenesis experiments confirmed that *tdm* is essential for growth on TMAO. We also identified a unique ABC transporter (TmoXWV) found in a variety of marine bacteria and experimentally confirmed its specificity for TMAO through marker exchange mutagenesis and *lacZ* reporter assays of the promoter for genes encoding this transporter. Both Tdm and TmoXWV are particularly abundant in natural seawater assemblages and actively expressed, as indicated by a number of recent metatranscriptomic and metaproteomic studies. These data suggest that TMAO represents a significant yet overlooked nutrient for marine bacteria.

Keywords: Trimethylamine *N*-oxide | TMAO transporter | TMAO demethylase | Marine *Roseobacter* Clade | SAR11 clade

Significance

Trimethylamine *N*-oxide (TMAO) is a nitrogen-containing osmolyte found in a wide variety of marine biota and has been detected at nanomolar concentrations in surface seawaters. This study provides the first genetic and biochemical evidence for uptake and catabolism of TMAO by marine heterotrophic bacteria that are abundant in the oceans. The genes conferring the ability of bacteria to catabolize TMAO we identified in this study are highly expressed in the marine environment and can be used as functional biomarkers to better understand oceanic microbial-mediated carbon and nitrogen cycles. Our data suggest that TMAO represents a significant, yet overlooked nutrient for marine bacteria in the surface oceans.

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Introduction

Trimethylamine *N*-oxide (TMAO) frequently occurs in the tissues of a variety of marine biota (1) and is predicted to have a number of important physiological roles (2). In marine elasmobranchs (sharks and rays), TMAO accumulates at high concentrations (up to 500 mM), helping to offset the destabilising effects of urea on cellular proteins (1, 3, 4). TMAO can be metabolised to small methylated amines, *e.g.* tri-, di-, and mono-methylamine, TMA, DMA, MMA, respectively. These volatile organic nitrogen compounds are precursors of marine aerosols and the potent greenhouse gas, nitrous oxide, in the marine atmosphere (5). In anoxic sediments or pockets of hypoxic conditions, such as in marine snow, they are precursors for the potent greenhouse gas, methane (6). In marine surface waters, TMAO concentrations can reach up to 79 nM, however, due to the technical difficulties associated with quantifying TMAO in seawater, reports of *in situ* concentration of TMAO are limited (7, 8). In a previously published study where TMAO and TMA have been quantified in the marine environment, TMAO had a higher average concentration throughout the water column and over a seasonal cycle (7).

TMAO is a well-studied terminal electron acceptor for anaerobic microbial respiration (9, 10) but its catabolism in aerobic surface seawater is not well understood. Recent studies have shown that TMAO in the Sargasso Sea is predominantly oxidised by bacterioplankton as an energy source (11) and that the marine methylotrophic bacterium, *Methylophilales* sp. HTCC2181, oxidises TMAO to CO_2 in order to generate energy (12). However, the genes and enzymes responsible for the metabolism and uptake of TMAO by marine bacteria are not known. It has previously been suggested that in *Methylocella silvestris*, a TMA-degrading soil bacterium, an aminotransferase protein containing a conserved C-terminal tetrahydrofolate (THF) binding domain (Msil_3603) is probably involved in the metabolism

of TMAO because this polypeptide was highly enriched in TMA-grown cells and TMAO is a known intermediate of TMA metabolism by TMA monooxygenase, Tmm, in this bacterium. ($TMA + NADPH + O_2 + H^+ \rightarrow TMAO + H_2O + NADP^+$) (13). It is hypothesized that TMAO is further metabolised to ammonium and formaldehyde, which serves as nitrogen and carbon/energy sources, respectively, for this bacterium (13).

ATP-binding cassette (ABC) transporters form one of the largest gene superfamilies found within many bacterial genomes (14) and their expression is frequently detected in the marine environment (15-17). ABC transporters are essential for bacteria because they are responsible for the uptake of a wide range of compounds, such as sugars, amino acids, metals and vitamins, at the expense of ATP (18). They usually consist of three sub-units: a transmembrane domain which is bound to an inner membrane-bound ATP-binding domain and a periplasmic substrate-binding protein (SBP), which binds a given ligand. SBPs confer substrate specificity and can bind their ligands with very high affinity (19, 20). One group of ABC transporters specialise in the uptake of compatible osmolytes and structurally-related compounds, such as glycine betaine (GBT), choline, carnitine, and proline betaine (21, 22). These transporters either function in osmoregulation (23), or play a role in substrate catabolism (19). A bacterial ATP-dependent TMAO transporter has been identified (24), but the genes encoding this transport system are unknown.

The SAR11 clade (*Pelagibacteraceae*) and the marine *Roseobacter* clade (MRC, *Rhodobacteraceae*) are two groups of marine bacteria which differ in their ecology but both play important roles in marine carbon, sulfur and nitrogen (N) cycles (25-27). Bacteria of the SAR11 clade bacteria dominate low nutrient environments, have streamlined genomes, are generally slow-growing and have distinct auxotrophic requirements for certain compounds (28-30). In contrast, bacteria of the MRC have larger genomes, display high metabolic versatility, can live a particle-associated lifestyle and often represent a large proportion of the

metabolically active bacterial community in coastal oceans (25, 31-34). Ecologically-relevant representatives of the MRC are readily cultivated and amenable to genetic manipulation, thereby making them good model organisms to investigate bacterial ecophysiology in the marine environment. *Ruegeria pomeroyi* DSS-3, isolated off the coast of Oregon in the USA (35), is the best characterised model marine organism in this clade (32, 36-39).

Here, we identify a TMAO-specific microbial ABC transporter and the TMAO demethylase, Tdm (TMAO →DMA + formaldehyde), from key marine heterotrophs, including bacteria from the SAR11 clade and the MRC. This transporter and Tdm are highly expressed in the marine environment as indicated by a number of recent metatranscriptomic and metaproteomic studies. Therefore, our data suggest that TMAO is an important, yet overlooked, nutrient for marine bacteria.

Results

Identification and confirmation of a functional Tdm in *R. pomeroyi*

We used *R. pomeroyi* DSS-3 as the model organism to study TMAO metabolism. This bacterium can grow on methylated amines, including TMAO, as a sole N source (Figure 1a). In the genome sequence of *R. pomeroyi*, we identified an ORF (SPO1562), that has high sequence similarity (54%) to Msil_3603, the ORF predicted to encode the Tdm in *M. silvestris* (13). Sequence analysis has shown that both proteins contain an uncharacterised domain (DUF1989) and a THF-binding domain, which is likely to be important in conjugating formaldehyde released from the demethylation of TMAO. In a representative of the SAR11 clade, it has been suggested that TMAO demethylation through THF-mediated one-carbon oxidation provides cellular energy (11). To confirm that SPO1562 in *R. pomeroyi* encodes for a *bona fide* Tdm, this gene was cloned and over-expressed in *Escherichia coli*. In

123 the presence of TMAO, *E. coli* cells expressing the putative Tdm from *R. pomeroyi* produced
124 984 ± 45 µM of DMA in the culture medium (Figure 2b), confirming that SPO1562 does
125 indeed encode for a Tdm. *E. coli* cells transformed with vector, pET28a alone, did not
126 produce DMA.

127 In order to determine if SPO1562 is required for growth of *R. pomeroyi* on TMAO, this gene
128 was mutated. As predicted, the mutant (*Δtdm::Gm*) could not grow on TMAO or its upstream
129 precursor TMA (Figure 1b) although it could grow on DMA and MMA (Table S1). To
130 confirm if *tdm* is essential in *R. pomeroyi*, *tdm* was cloned along with its promoter from *R.*
131 *pomeroyi* into the broad-host range plasmid, pBBR1MCS-km (40), which was then mobilised
132 into the *Δtdm::Gm* mutant *via* conjugation. Complementation of the mutant with the native
133 *tdm* gene from *R. pomeroyi* reversed the phenotype, restoring growth on both TMAO and
134 TMA as a sole N source (Figure 1d). Complementation of this mutant with the vector
135 pBBR1MCS-km alone did not result in growth on TMA and TMAO (Figure 1c).

136 **Distribution of Tdm homologs in other marine bacteria**

137 To test the importance of the *tdm* gene and to investigate its occurrence in the marine
138 environment, we further investigated the distribution of Tdm in the genomes of isolated
139 marine bacteria (41). The Tdm from *R. pomeroyi* was used as the query sequence to generate
140 a BLASTP database using the Integrated Microbial Genomes (IMG) system at the Joint
141 Genome Institute. Closely related homologs (E value = 0.0) of Tdm were retrieved from
142 representatives of the SAR11 clade and the MRC of the *Alphaproteobacteria*, the SAR324
143 cluster of *Deltaproteobacteria* and some *Gammaproteobacteria* (Figure 2a, Figure S1). In
144 general, the presence of *tmm*, the gene encoding TMA monooxygenase, coincides with the
145 presence of *tdm,* but not *vice* versa. Those bacteria lacking *tmm* do, however, have the genes
146 necessary for further downstream catabolism of MMA (13, 26). One example is *Roseobacter*

147 sp. SK209-2-6, a representative of the MRC. This bacterium lacks *tmm* in its genome but
148 does contain *tdm* and genes required for MMA catabolism (*e.g. gmaS*) (26). As predicted,
149 *Roseobacter* sp. SK209-2-6 failed to grow on TMA but could grow on TMAO (Table S2).

150 We generated another BLASTP database using the Global Ocean Sampling (GOS)
151 Expedition database (41) and we estimated that Tdm homologs are present in 21% of
152 bacterial cells inhabiting surface seawater, comparable to estimates for Tmm (20%) and
153 GmaS (23%) (13). Tdm sequences were present in both open ocean and coastal ocean surface
154 waters (Figure S2). Phylogenetic analysis indicated that majority of Tdm homologs (92%)
155 identified from the GOS dataset were related to the Tdm of the SAR11 clade, and the
156 remaining were related to the MRC (5%), *Gammaproteobacteria* (2%) and
157 *Deltaproteobacteria* (1%).

158 Tdm homologs from representatives of the SAR11 clade share ~57% sequence similarity at
159 the amino acid level to the Tdm from *R. pomeroyi* DSS-3. As yet, no genetic system has
160 been established for SAR11 strains, so in order to confirm that these Tdm homologs are
161 functional, a Tdm homolog from the SAR11 clade representative, *Pelagibacteraceae* strain
162 HIMB59, was cloned and over-expressed in *E. coli*. In the presence of TMAO, *E. coli* cells
163 expressing Tdm produced 171 ± 34 μM DMA (Figure 2b). Complementation of the *R.*
164 *pomeroyi* mutant (*Δtdm::Gm*) with the native *tdm* homolog from *Pelagibacteraceae* strain
165 HIMB59 also reversed the phenotype (Figure 1e). These experiments suggest that the SAR11
166 *tdm* homologs also encode a functional Tdm.

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168 **Identification and characterisation of a novel TMAO-specific ABC transporter**

The fact that some bacteria, such as *Roseobacter* sp. SK209-2-6 can metabolise TMAO but not TMA suggests that TMAO transport into the cell can be independent of TMA metabolism. This led us to hypothesise that a specific transporter for TMAO is needed for such microorganisms. We therefore systematically investigated the presence of membrane transporter proteins in the genomes of marine bacteria possessing a Tdm and paid particular attention to the neighborhoods of genes known to be involved in methylated amine metabolism, *e.g. tdm, tmm, gmaS*. We found a conserved three-ORF gene cluster encoding a putative GBT/ proline betaine ABC transporter present in the neighborhood of *tdm* in many marine bacterial genomes, including *Roseobacter* sp. SK209-2-6 (Figure 3). These genes encode a periplasmic SBP, an ATP-binding domain protein and a transmembrane permease protein, and are hereafter designated as *tmoX, tmoW, and tmoV,* respectively. In some MRC bacteria (*Roseovarius* sp. 217, *Roseovarius* sp. TM1035 and *Roseobacter* sp. Azwk-3B), this *tmoXWV* gene cluster is located adjacent to genes encoding a two-component regulatory system, *torRTS*. These regulatory proteins are known to be involved in the regulation of the TMAO reductase in *E. coli,* which is required for anaerobic respiration of TMAO (10, 42). None of these three MRC bacteria possess a TMAO reductase homolog and we therefore conclude that these two gene clusters are involved in aerobic catabolism of TMAO. Our conclusion is further supported by phylogenetic analysis of the SBPs of the GBT/ proline betaine-type ABC transporter family. TmoX is part of the cluster F III of the ABC transporter superfamily, containing SBPs specific for compatible osmolytes (22). However, TmoX forms a distinct subcluster within cluster F III which does not contain any previously characterised SBPs (Figure 4). Other GBT/ proline betaine-type SBPs from *R. pomeroyi*, *Roseovarius* sp. 217, *Pelagibacteraceae* strain HIMB59 and *Candidatus* Pelagibacter ubique sp. HTCC1002/ HTCC1062 fall within the traditional F III subcluster (Figure S3).

The *tmoXWV* gene cluster (SPO1548-SPO1550) was targeted for mutagenesis again, using *R. pomeroyi* as a model bacterium. Two transporter mutants were generated; one targeting both *tmoX* and *tmoW* to mutate the entire membrane component of the transporter (*ΔtmoXW::Gm*) and the other targeting only the periplasmic SBP (*ΔtmoX::Gm*) leaving the core transporter domain intact. Growth on TMAO as a sole N source was significantly reduced for mutants, *ΔtmoX::Gm* (Figure 5) and *ΔtmoXW::Gm* (Figure S4). Over 96 hr, wild-type cells metabolised over 1 mM of TMAO whilst the two mutants only metabolised 87 ± 14 µM of added TMAO (Figure 5 a, c, Figure S4). The growth of the mutants on TMA, however, was unaffected (Figure 5b, Figure S4) suggesting that this transporter is only involved in TMAO but not TMA metabolism. Complementation of the *ΔtmoX::Gm* mutant with the native *tmoX* from *R. pomeroyi* reversed the phenotype (Figure 5c).

To better understand the specificity of this transporter, the transporters mutants (*ΔtmoXW::Gm* and *ΔtmoX::Gm*) were tested for their growth on structurally-related compounds (GBT, choline and carnitine), as a sole N source. Growth rates of the mutants (*ΔtmoXW::Gm* and *ΔtmoX::Gm*) were unaffected when grown on these three osmolytes and TMA (Figure 6a). We probed the transcriptional specificity of the promoter of the *tmoXWV* gene cluster in *R. pomeroyi*. The promoter of *tmoXWV* (~250 bp upstream region) was cloned into the broad host-range promoter probe vector, pBIO1878 (36), upstream of its *lacZ* reporter region. The resulting plasmid pBIOIL101 was mobilized into *R. pomeroyi* DSS-3 and a transconjugant was grown overnight in minimal medium either lacking any osmolyte or containing GBT, choline, carnitine, or TMAO (3 mM), prior to assaying for *β*-galactosidase activity. The presence of TMAO led to a 6-fold increase in induction of the *tmoX-lacZ* fusion whilst no induction was observed with the other osmolytes tested (Figure 6b). TMA also led to the induction of the transporter (Figure S5), however we hypothesised that intracellular production of TMAO through TMA oxidation was responsible for this phenomenon. To test

this hypothesis, we mobilized the pBIOIL101 plasmid into the mutant *Δtmm::Gm,* which can no longer grow on TMA as a sole nitrogen source (Figure S5). In this strain, TMAO still induced the transporter, however, the sensitivity of the transporter to TMA was significantly reduced (Fig S5). Together, these data suggest that the ABC-transporter *tmoXWV* is specific for TMAO and is essential for TMAO metabolism in *R. pomeroyi* DSS-3.

Discussion

We report the identification of the genes encoding the Tdm and a TMAO-specific ABC transporter in a number of divergent marine bacteria, including MRC and SAR11 clade *Alphaproteobacteria*, SAR324 clade *Deltaproteobacteria* and some *Gammaproteobacteria* (Figures 2-4). The Tdm and the associated TMAO transporter and the genes encoding these proteins are widespread in both coastal and open ocean surface seawater and we estimate using the GOS metagenome dataset that one in five bacterial cells are capable of TMAO catabolism (Figure S2). It is noteworthy that Tdm and TmoXWV are found not only in cultivated representatives of abundant marine bacteria (*e.g.* SAR11 and MRC), but also in as-yet uncultivated marine bacteria inhabiting the surface Oceans with streamlined genomes (Figure S1, S3). For example, these genes are found in single-cell amplified genomes of uncultivated *Roseobacters* that are prevalent in tropical and temperate regions of the Oceans (AAA298-K06) as well as in Polar Oceans (AAA076-C03) (53).

The ability to utilise the potentially more abundant TMAO directly from the water column would provide an energetic and ecological benefit to marine bacteria. Conversely, the conversion of TMA to TMAO requires an extra enzyme and NADPH as a reducing equivalent and production of TMA is reliant on the anaerobic conversion of quaternary

amines, including TMAO, and may not be relevant to open ocean systems. Our study has shown that some bacteria do not have the genetic potential to metabolise TMA but are still able to metabolise TMAO (*e.g. Roseobacter* sp. SK209-2-6). In addition, all Tdm-containing marine bacteria have a TMAO-specific transporter, thereby strengthening the hypothesis that TMAO is an important nutrient in the marine environment and not simply an intermediate of intracellular TMA metabolism, as proposed previously (13). This hypothesis is supported by at least three key observations. Firstly, TMAO is directly produced in a diverse range of marine biota and has been detected in marine surface seawater (2, 8). Secondly, TMAO added to surface seawater can be metabolised to CO_2 by marine microorganisms to generate cellular energy (11). Thirdly, re-analyses of a number of recent metatranscriptomic and metaproteomic datasets has indicated that Tdm and the newly identified TMAO-specific ABC transporter are highly expressed *in situ* (15, 17, 43-45). For example, analysis of metatranscriptomic data of bacterioplankton from the Monterey Bay of California showed that the TMAO transporter is one of the most highly expressed transporters in the MRC representative, *Rhodobacterales* sp. HTCC2255 (ZP_01447069), an abundant member of the microbial community (17), whilst off the coast of northern California, *tmoX* from SAR11 bacteria (Cluster 686, YP_266709) is among the 10 most highly expressed genes (43). Metaproteomic data collected from the Sargasso Sea also revealed that a polypeptide identified as TmoX, closely related to TmoX of the SAR11 isolate *Candidatus* Pelagibacter sp. 7211 (PB7211_687), was among the 10 most highly expressed transporter proteins (15). During the summer and winter months in Antarctic surface seawater, a TmoX, closely related to the TmoX of *Candidatus* Pelagibacter ubique HTCC1002 (PU1002_06741), was also highly expressed (45). Not only has expression of the TMAO transporter been frequently detected in natural seawater by metatranscriptomic and metaproteomic studies, but Tdm expression (Cluster 435, YP_266710) has also been found in bacterial plankton assemblages

in the surface seawater (43). The high level of *tmoX* and *tdm* expression in SAR11 and MRC bacteria from natural bacterioplankton communities points towards TMAO serving as an important substrate for energy generation (11) and may also be an important source of N for these heterotrophs in the marine environment.

Several lines of evidence further suggest that the metabolism of TMAO is important in the marine environment. For example, a *tmm* homolog is present in the genome of the marine N fixer *Trichodesmium erythraeum* IMS101. Whilst there are no data regarding the function of Tmm or whether TMAO has any physiological role in *Trichodesmium*, a MRC bacterium, *Roseibium* sp. TrichSDK4, isolated from *Trichodesmium* colonies, has the genes necessary for TMAO catabolism but lacks a Tmm. It is therefore tempting to speculate that this bacterium may benefit from TMAO released by *Trichodesmium* cells. We also found Tdm and the TMAO transporter in the genome of a SAR324 cluster bacterium, which is predominantly found in the deep ocean "twilight zone" where photosynthesis does not occur (46-47). TMAO metabolism by SAR324 bacteria may help facilitate their chemoautotrophic lifestyle, supplementing energy predominantly derived from the oxidation of reduced sulfur compounds (46). Genes required for the THF-linked oxidation of methyl groups cleaved off during the dissimilation of TMAO were indeed expressed among the SAR324 cluster bacteria inhabiting deep sea marine plumes (47). The ability of SAR324 bacteria to use TMAO is in line with the recent discovery that they are capable of utilising a range of electron donors and acceptors, which helps explain their prevalence in the dark ocean (47).

We noticed that both transporter mutants (*ΔtmoXW::Gm* and *ΔtmoX::Gm*) can still deplete TMAO from the medium, albeit at much slower rates (Figure 5, Figure S4), suggesting the presence of another yet-undiscovered membrane transporter for TMAO. Indeed, in the genome of *Methylocella silvestris* (13), no homologs of *tmoXWV* were found although it can utilize TMAO as a sole nitrogen source. It is also likely that in *R. pomeroyi*, there is a SBP of

broad specificity but lower affinity for TMAO, therefore contributing to the slower growth rates on TMAO observed in the mutants, and clearly this warrants further investigation. We cannot rule out the possibility that TmoXWV may also serve as a high affinity TMA transporter and further investigation is required to determine the affinity of this transporter for both TMA and TMAO. In *A. aminovorans*, a high concentration of TMA (5 mM) only partially inhibited uptake of TMAO (at 10µM) and it was proposed that there might be two different high affinity transporters for these two compounds (24). As we observed no difference in TMA metabolism in the mutant, *ΔtmoX::Gm*, we also propose that in ***R. pomeroyi***, another high affinity transport system is necessary for TMA uptake. Alternative microbial pathways for TMAO catabolism in surface seawaters are also likely. For example, *Methylophilales* sp. HTCC2181, lacks the *tdm* gene required for TMAO metabolism, however it can oxidise TMAO to CO_2, as demonstrated previously (12). Similarly, multiple enzymes responsible for the cleavage of the compatible osmolyte, dimethylsulfoniopropionate into the climate-active gas, dimethylsulfide, have now been identified (36, 48).

In conclusion, our discovery of the genes encoding the TMAO demethylase and a TMAO-specific ABC transporter in abundant members of the bacterioplankton, and the prevalence of these genes and their transcription and subsequent expression in natural surface seawaters implies that this compound is an important nutrient for different groups of heterotrophic bacteria in the marine environment.

Materials and Methods

Cultivation of MRC bacteria on methylamines.

MRC bacteria were grown at 30 °C in 125-ml serum vials in triplicate using a defined medium as previously described (13). Methylated amines (0.5 mM) were used as the sole N source. Succinate (5-10 mM) was used as the sole carbon source. Vitamins were added as described previously (13). To test if the TMAO demethylase mutant (*Δtdm::Gm*) and the TMAO ABC transporter mutants (*ΔtmoXW::Gm*, *ΔtmoX::Gm*) mutants could grow on methylated amines, growth experiments were set up in triplicate using 120-mL serum vials, containing 20 ml medium with an inoculum size of 10%.

Marker exchange mutagenesis and complementation of *R. pomeroyi* mutants.

All strains used for cloning are listed in Table S3. All primers used for PCR and sequencing are listed in Table S4. The method for marker exchange mutagenesis was modified from (49). Detailed protocols for marker exchange mutagenesis and complementation of mutants in *R. pomeroyi* are described in SI Material and Methods.

Overexpression of Tdm in *Escherichia coli.*

The *tdm* gene from *R. pomeroyi* DSS-3 was amplified by PCR (primers used are listed in Table S4) and cloned into the expression vector pET28a (Merck Biosciences). The *tdm* gene from *Pelagibacteraceae* strain HIMB59 was chemically synthesized (GenScript Corporation) and cloned into pET28a. The resulting plasmids were transformed into the expression host *Escherichia coli* BLR(DE3) pLysS (Merck Biosciences). Detailed protocols for protein expression and DMA quantification are described in SI Material and Methods.

Identification of Tmm and GmaS homologs in the GOS metagenome.

The Tdm and Tmm sequences of *R. pomeroyi* were used as query sequences for a BLASTP search of the GOS peptides at CAMERA [https://portal.camera.calit2.net/gridsphere/gridsphere?cid=]; GOS: all ORF peptides (P) database [e−60], and this resulted in 2,274 and

1,177 unique sequences, respectively. For Tdm, sequences were further grouped into 122 unique groups (identity >80% within each group) using the CD-HIT program (48). Representative sequences from each group were aligned using MEGA 5.1 (49). To estimate the frequency of Tdm-containing cells, the data were processed as described previously (13, 50). To compare the distribution of Tdm and Tmm against each other in the GOS dataset, both proteins were normalised to RecA by the following: Tdm =RecA (376)/ Avg. Tdm length (778); Tmm = RecA (376)/Avg Tmm length (445). The number of reads at each site were normalised per 100,000 reads.

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References

1. Yancey PH, Clark ME, Hand SC, Bowlus RD, & Somero GN (1982) Living with water stress: evolution of osmolyte systems. *Science* 217(4566):1214-1222.
2. Seibel BA & Walsh PJ (2002) Trimethylamine oxide accumulation in marine animals: relationship to acylglycerol storage. *J Exp Biol* 205(3):297-306.
3. Ballantyne JS (1997) Jaws: the inside story. the metabolism of elasmobranch fishes. *Comp Biochem Physiol B Biochem Mol Biol* 118(4):703-742.
4. Treberg JR, *et al.* (2006) The accumulation of methylamine counteracting solutes in elasmobranchs with differing levels of urea: a comparison of marine and freshwater species. *J Exp Biol* 209(5):860-870.
5. Quinn PK, Charlson RJ, & Bates TS (1988) Simultaneous observations of ammonia in the atmosphere and ocean. *Nature* 335(6188):336-338.
6. King GM (1984) Metabolism of trimethylamine, choline, and glycine betaine by sulfate-reducing and methanogenic bacteria in marine sediments. *Appl Environ Microbiol* 48(4):719-725.
7. Carpenter LJ, Archer SD, & Beale R (2012) Ocean-atmosphere trace gas exchange. *Chem Soc Rev*41(19):6473-6506.
8. Gibb SW & Hatton AD (2004) The occurrence and distribution of trimethylamine-*N*-oxide in Antarctic coastal waters. *Marine Chemistry* 91(1–4):65-75.
9. Arata H, Shimizu M, & Takamiya K (1992) Purification and properties of trimethylamine *N*-oxide reductase from aerobic photosynthetic bacterium *Roseobacter denitrificans*. *J Biochem* 112(4):470-475.
10. Gon S, Giudici-Orticoni M-T, Méjean V, & Iobbi-Nivol C (2001) Electron transfer and binding of the c-type cytochrome TorC to the trimethylamine *N*-oxide reductase in *Escherichia coli*. *J Biol Chem* 276(15):11545-11551.

11. Sun J, *et al.* (2011) One carbon metabolism in SAR11 pelagic marine bacteria. *PLoS ONE* 6(8):e23973.
12. Halsey KH, Carter AE, & Giovannoni SJ (2012) Synergistic metabolism of a broad range of C1 compounds in the marine methylotrophic bacterium HTCC2181. *Environ Microbiol* 14(3):630-640.
13. Chen Y, Patel NA, Crombie A, Scrivens JH, & Murrell JC (2011) Bacterial flavin-containing monooxygenase is trimethylamine monooxygenase. *Proc Natl Acad Sci USA* 108(43):17791-17796.
14. Young J & Holland IB (1999) ABC transporters: bacterial exporters-revisited five years on. *Biochim Biophys Acta (BBA) - Biomembranes* 1461(2):177-200.
15. Sowell SM, *et al.* (2008) Transport functions dominate the SAR11 metaproteome at low-nutrient extremes in the Sargasso Sea. *ISME J* 3(1):93-105.
16. Sowell SM, *et al.* (2011) Environmental proteomics of microbial plankton in a highly productive coastal upwelling system. *ISME J* 5(5):856-865.
17. Ottesen EA, *et al.* (2011) Metatranscriptomic analysis of autonomously collected and preserved marine bacterioplankton. *ISME J* 5(12):1881-1895.
18. Davidson AL & Chen J (2004) ATP-binding cassette transporters in bacteria. *Annu Rev Biochem* 73(1):241-268.
19. Chen C, Malek AA, Wargo MJ, Hogan DA, & Beattie GA (2010) The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds. *Mol Microbiol* 75(1):29-45.
20. Albers SV, *et al.* (1999) Glucose transport in the extremely thermoacidophilic *Sulfolobus solfataricus* involves a high-affinity membrane-integrated binding protein. *J Bacteriol* 181(14):4285-4291.

21. Thomas GH (2010) Homes for the orphans: utilization of multiple substrate-binding proteins by ABC transporters. *Mol Microbiol* 75(1):6-9.

22. Berntsson RPA, Smits SHJ, Schmitt L, Slotboom D-J, & Poolman B (2010) A structural classification of substrate-binding proteins. *FEBS Letters* 584(12):2606-2617.

23. May G, Faatz E, Villarejo M, & Bremer E (1986) Binding protein dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K12. *Mol Gen Genet* 205(2):225-233.

24. Raymond JA & Plopper GE (2002) A bacterial TMAO transporter. *Comp Biochem Physiol B Biochem Mol Biol* 133(1):29-34.

25. Buchan A, González JM, & Moran MA (2005) Overview of the marine *Roseobacter* lineage. *Appl Environ Microbiol* 71(10):5665-5677.

26. Chen Y (2012) Comparative genomics of methylated amine utilization by marine *Roseobacter* clade bacteria and development of functional gene markers (*tmm, gmaS*). *Environ Microbiol* 14(9):2308-2322.

27. Tripp HJ, *et al.* (2008) SAR11 marine bacteria require exogenous reduced sulphur for growth. *Nature* 452(7188):741-744.

28. Giovannoni SJ, *et al.* (2005) Genome streamlining in a cosmopolitan oceanic bacterium. *Science* 309(5738):1242-1245.

29. Tripp HJ, *et al.* (2009) Unique glycine-activated riboswitch linked to glycine–serine auxotrophy in SAR11. *Environ Microbiol* 11(1):230-238.

30. Grote J, *et al.* (2012) Streamlining and core genome conservation among highly divergent members of the SAR11 Clade. *MBio* 3(5).

31. Newton RJ, *et al.* (2010) Genome characteristics of a generalist marine bacterial lineage. *ISME J* 4(6):784-798.

426 32. Moran MA, *et al.* (2004) Genome sequence of *Silicibacter pomeroyi* reveals
427 adaptations to the marine environment. *Nature* 432(7019):910-913.
428 33. Wagner-Döbler I, *et al.* (2009) The complete genome sequence of the algal symbiont
429 *Dinoroseobacter shibae*: a hitchhiker's guide to life in the sea. *ISME J* 4(1):61-77.
430 34. Alonso C & Pernthaler J (2006) *Roseobacter* and SAR11 dominate microbial glucose
431 uptake in coastal North Sea waters. *Environ Microbiol* 8(11):2022-2030.
432 35. González JM, *et al.* (2003) *Silicibacter pomeroyi* sp. nov. and *Roseovarius*
433 *nubinhibens* sp. nov., dimethylsulfoniopropionate-demethylating bacteria from marine
434 environments. *Int J Syst Evol Microbiol* 53(5):1261-1269.
435 36. Todd JD, Kirkwood M, Newton-Payne S, & Johnston AWB (2012) DddW, a third
436 DMSP lyase in a model *Roseobacter* marine bacterium, *Ruegeria pomeroyi* DSS-3.
437 *ISME J* 6(1):223-226.
438 37. Sebastian M & Ammerman JW (2011) Role of the phosphatase PhoX in the
439 phosphorus metabolism of the marine bacterium *Ruegeria pomeroyi* DSS-3. *Environ*
440 *Microbiol Rep* 3(5):535-542.
441 38. Cunliffe M (2012) Physiological and metabolic effects of carbon monoxide oxidation
442 in the model marine bacterioplankton *Ruegeria pomeroyi* DSS-3. *Appl Environ*
443 *Microbiol* 79(2):738-740.
444 39. Christie-Oleza JA, Fernandez B, Nogales B, Bosch R, & Armengaud J (2012)
445 Proteomic insights into the lifestyle of an environmentally relevant marine bacterium.
446 *ISME J* 6(1):124-135.
447 40. Kovach ME, *et al.* (1995) Four new derivatives of the broad-host-range cloning vector
448 pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166(1):175-176.
449 41. Rusch DB, *et al.* (2007) The Global Ocean Sampling Expedition: Northwest Atlantic
450 through Eastern Tropical Pacific. *PLoS Biol* 5(3):e77.

42. Gon S, Patte J-C, Dos Santos J-P, & Méjean V (2002) Reconstitution of the trimethylamine oxide reductase regulatory elements of *Shewanella oneidensis* in *Escherichia coli*. *J Bacteriol* 184(5):1262-1269.

43. Ottesen EA, *et al.* (2013) Pattern and synchrony of gene expression among sympatric marine microbial populations. *Proc Natl Acad Sci USA*: 110 (6) E488-E497.

44. Gifford SM, Sharma S, Booth M, & Moran MA (2013) Expression patterns reveal niche diversification in a marine microbial assemblage. *ISME J* 7(2):281-298.

45. Williams TJ, *et al.* (2012) A metaproteomic assessment of winter and summer bacterioplankton from Antarctic Peninsula coastal surface waters. *ISME J* 6(10):1883-1900.

46. Swan BK, *et al.* (2011) Potential for chemolithoautotrophy among ubiquitous Bacteria lineages in the dark ocean. *Science* 333(6047):1296-1300.

47. Sheik CS, Jain S, & Dick GJ (2013) Metabolic flexibility of enigmatic SAR324 revealed through metagenomics and metatranscriptomics. *Environ Microbiol*: doi: 10.1111/1462-2920.12165

48. Kirkwood M, Le Brun NE, Todd JD, & Johnston AWB (2010) The *dddP* gene of *Roseovarius nubinhibens* encodes a novel lyase that cleaves dimethylsulfoniopropionate into acrylate plus dimethyl sulfide. *Microbiology* 156(6):1900-1906.

49. Crombie A & Murrell JC (2011) Development of a system for genetic manipulation of the facultative methanotroph *Methylocella silvestris* BL2. *Methods Enzymol* 495:119-133.

50. Howard EC, Sun S, Biers EJ, & Moran MA (2008) Abundant and diverse bacteria involved in DMSP degradation in marine surface waters. *Environ Microbiol* 10(9):2397-2410.

51. Tamura K, *et al.* (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28(10):2731-9

52. Saitou N & Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4(4):406-425.

53. Swan BK, *et al.* (2013) Prevalent genome streamlining and latitudinal divergence of planktonic bacteria in the surface ocean. *Proc Natl Acad Sci USA*: 110 (28):11463-11468.

Figure Legends

Figure 1. Growth of *Ruegeria pomeroyi* DSS-3 on trimethylamine (TMA) and trimethylamine *N*-oxide (TMAO) as a sole nitrogen source. *Ruegeria pomeroyi* DSS-3 was grown on either TMA (white circles) or TMAO (grey circles) and concentrations of TMA (white diamonds) and TMAO (grey diamonds) were quantified throughout the growth. The function of *tdm* was determined by comparing growth of the wild type **(a)** against the *Δtdm::Gm* mutant **(b).** When the mutant was corrected with a native *tdm* from either *R. pomeroyi* DSS-3 **(d)** or *Pelagibacteraceae* strain HIMB59 **(e),** growth was restored, whereas the vector control (pBBR1MCS-km) did not restore the growth of the mutant on TMA or TMAO (**c**). All cultures were grown in triplicate and error bars denote standard deviations.

Figure 2. a) Neighbour-Joining phylogenetic analysis of TMAO demethylase (Tdm) retrieved from the genomes of sequenced marine bacteria. Bootstrap values (500 replicates) greater than 60% are shown. The scale bar denotes the number of amino acid differences per site. The analysis involved 49 Tdm sequences. There were a total of 468 amino acid residues in the alignment. Evolutionary analyses were conducted in MEGA5.1 (51).
b) Production of dimethylamine (DMA) from TMAO demethylation by recombinant Tdm of *R. pomeroyi* DSS-3 and *Pelagibacteraceae* strain HIMB59. pET28a represents the control empty vector with no insert. Error bars denote standard deviations of triplicate measurements. IPTG, isopropyl β-D-1-thiogalactopyranoside.

Figure 3. Genetic neighbourhoods of the genes (*tmoXWV*) that encode the TMAO transporter (red) among representative genome-sequenced marine bacteria. All genes coloured black have no confirmed functional relationship with TMAO metabolism. Abbreviations: TMA,

trimethylamine; TMAO, trimethylamine *N*-oxide; NMG, *N*-methylglutamate, GMA, γ-glutamylmethylamide; α. *Alphaproteobacteria*; γ, *Gammaproteobacteria*; δ, *Deltaproteobacteria.*

Figure 4. Phylogenetic analysis of the substrate-binding protein (SBP), TmoX, of the TMAO-specific transporter in relation to other characterised SBPs. Current known SBPs specific for osmolytes, such as choline, glycine betaine and carnitine, fall into the Cluster F of the ATP-binding cassette (ABC) superfamily (22). The evolutionary history was inferred using the neighbour-joining method (52). Bootstrap values (500 replicates) greater than 99% are shown. The scale bar represents the number of amino acid differences per site. The analysis involved 69 SBP sequences. There were a total of 296 amino acids positions in the alignment. Evolutionary analyses were conducted in MEGA5.1 (51). Abbreviations; MRC, marine *Roseobacter* clade; δ, *Deltaproteobacteria*; γ, *Gammaproteobacteria*; TmoX, TMAO SBP; BetX, glycine betaine/ proline betaine SBP; CaiX, carnitine SBP; ChoX, choline SBP.

Figure 5. Growth of *Ruegeria pomeroyi* DSS-3 and the TMAO transporter mutants on trimethylamine (TMA) and trimethylamine *N*-oxide (TMAO) as a sole nitrogen source.
a) *R. pomeroyi* wild-type was grown on either TMA (grey circles) or TMAO (white circles) and concentrations of TMA (grey diamonds) and TMAO (white diamonds) were quantified during growth.
b) *R. pomeroyi* mutant *ΔtmoX::Gm* was grown on TMA (grey circles) and the concentration of TMA (grey diamonds) was quantified through the growth.
c) *R. pomeroyi* mutant *ΔtmoX::Gm* was grown on TMAO (white circles) and the concentration of TMAO (white diamonds) was quantified throughout growth. The mutant was complemented with the native *tmoX* from *R. pomeroyi* which was grown on TMAO as a

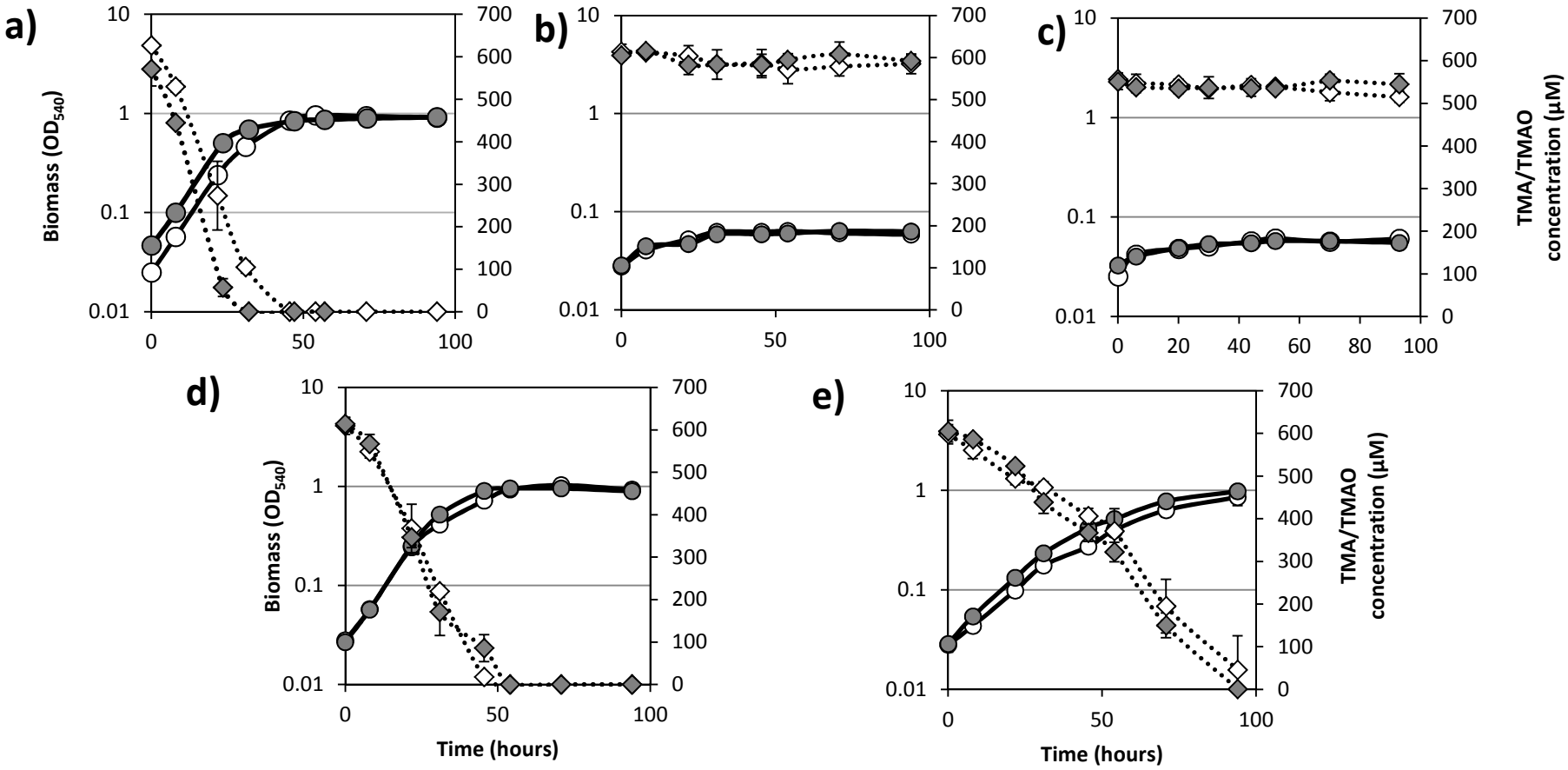
sole N source (white squares) and the concentration of TMAO was quantified (white triangles). Once TMA/TMAO was depleted in the medium, a second dose (final concentration 0.5 mM) was added at t=48 hr. All cultures were grown in triplicate and error bars denote standard deviations.

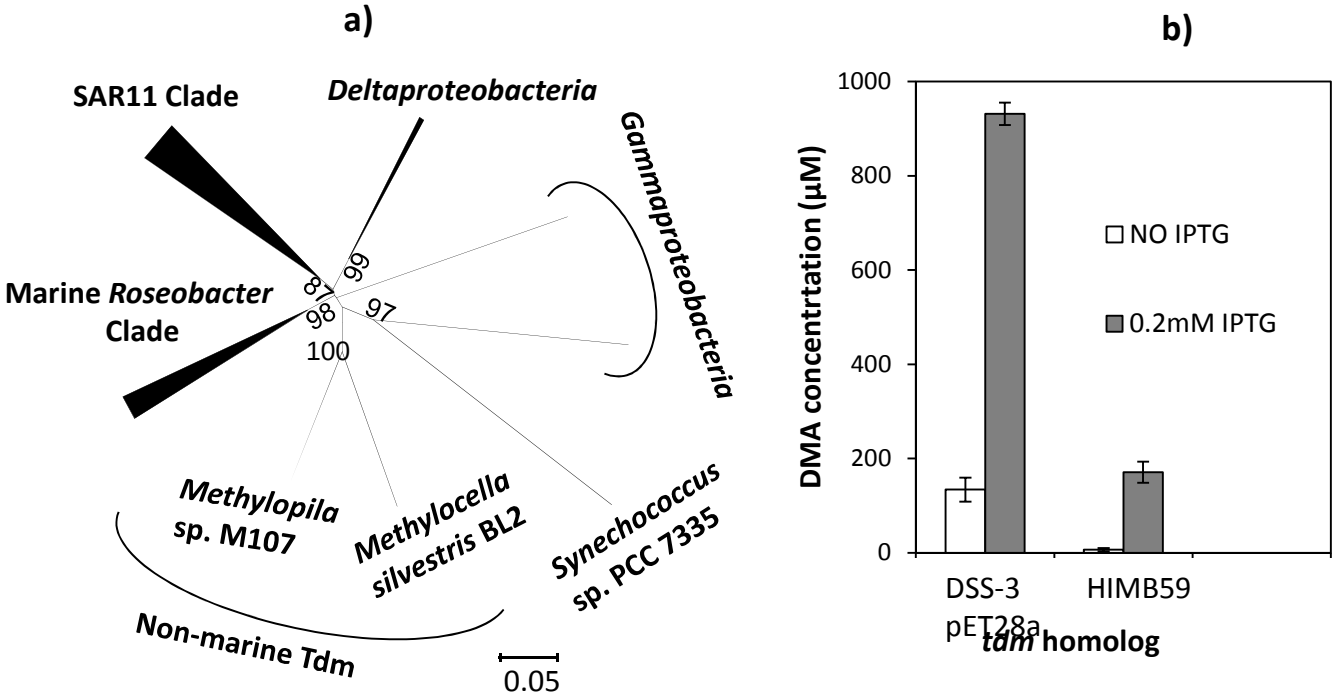
Figure 6. Effects of different compatible osmolytes on the growth of *Ruegeria pomeroyi* DSS-3 and regulation of the TMAO transporter, *tmoXWV*. The growth rates of *Ruegeria pomeroyi* wild type (white bars) and the two transporter mutants, *ΔtmoX::Gm* (grey bars) and *ΔtmoXW::Gm* (black bars), were determined for each osmolyte and TMA as a sole nitrogen source.

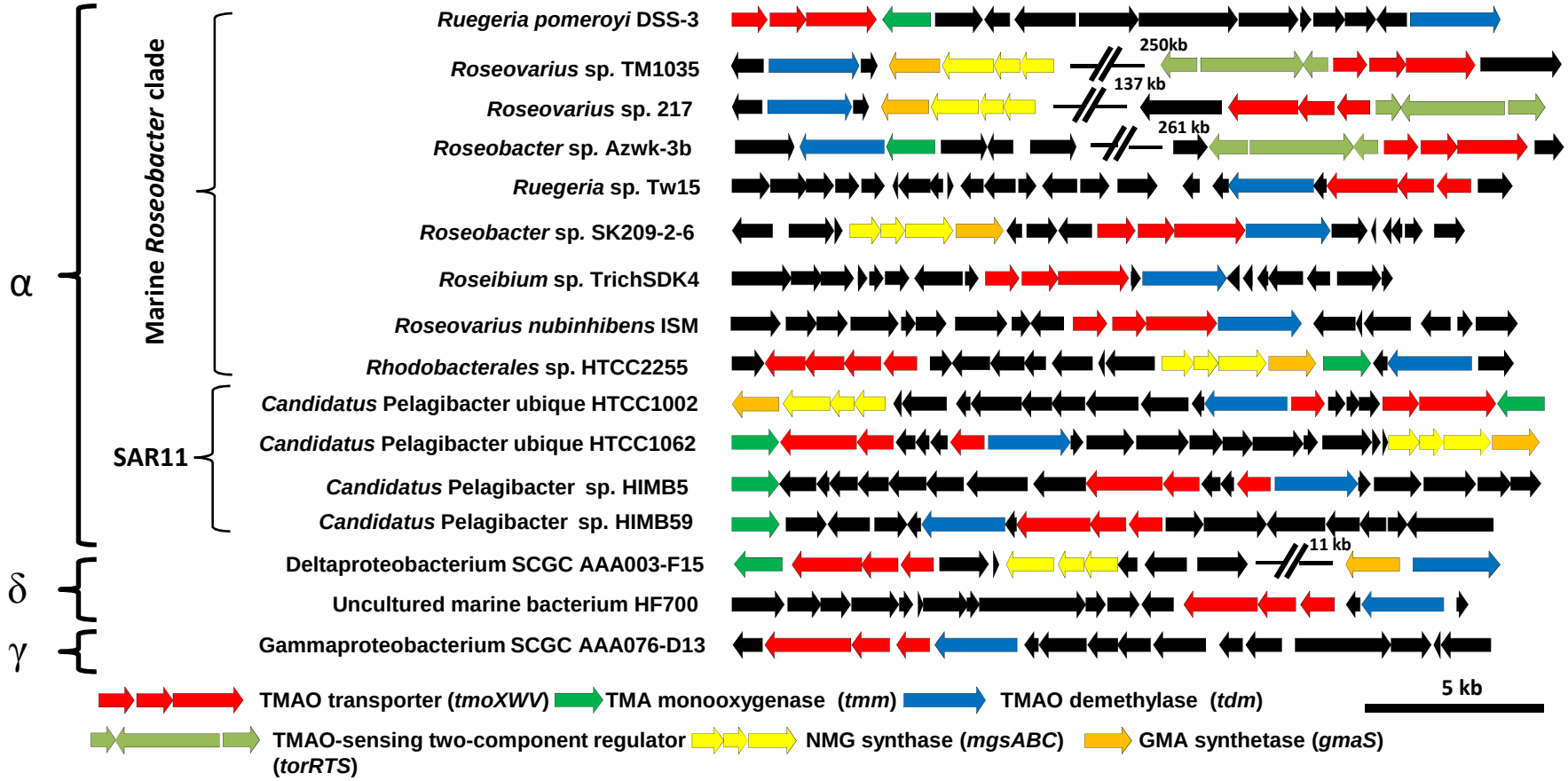
(a) Cultures of *Ruegeria pomeroyi* DSS-3 containing the *tmoX-lacZ* fusion plasmid, pBIL101 were grown in the presence of each compatible osmolyte (3 mM).

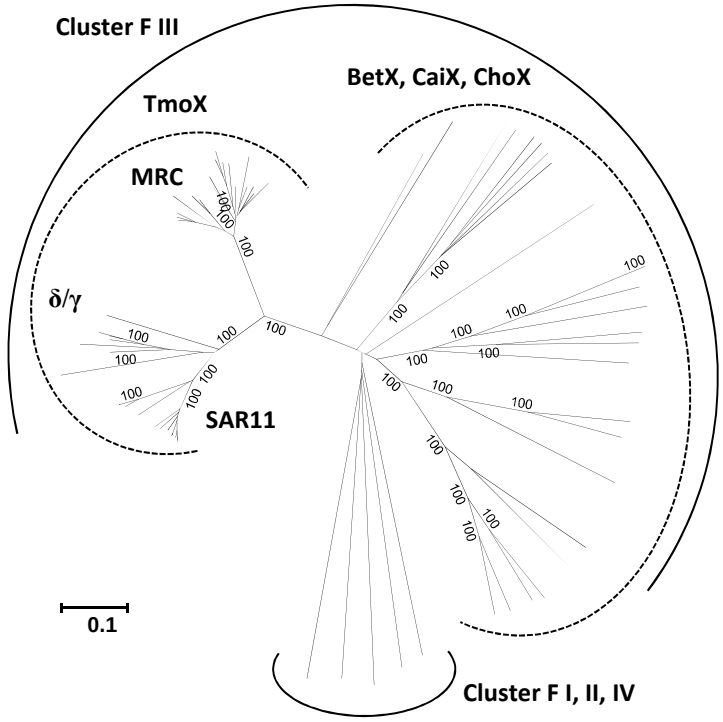
(b) Cultures were grown and assayed in triplicate for β-galactosidase activity and error bars denote standard deviations.

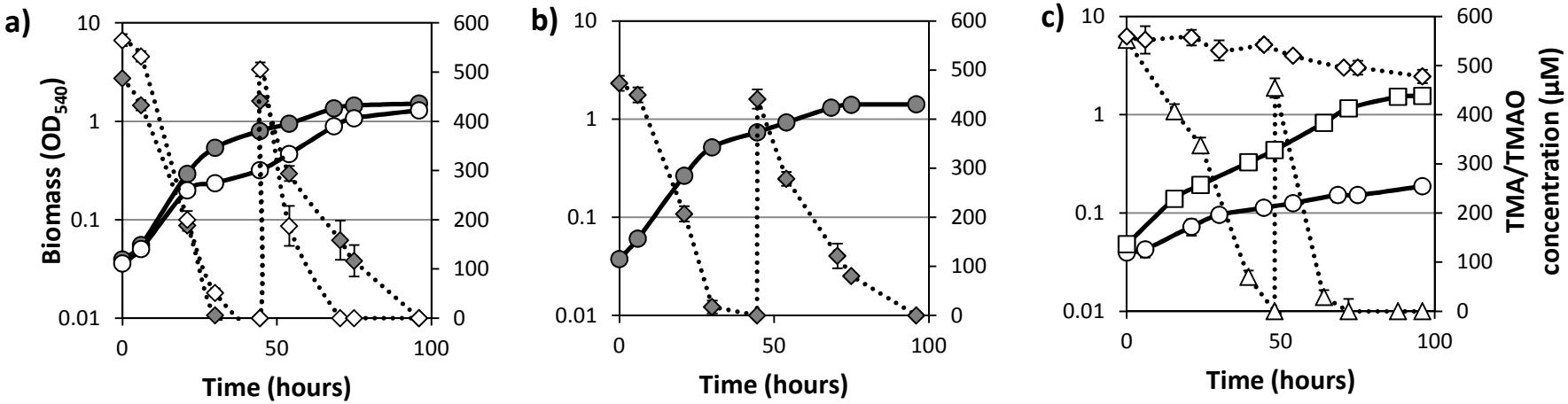
Abbreviations; Con, control; Cho, choline; GBT, glycine betaine, Car, carnitine; TMAO, trimethylamine *N*-oxide; TMA, trimethylamine.

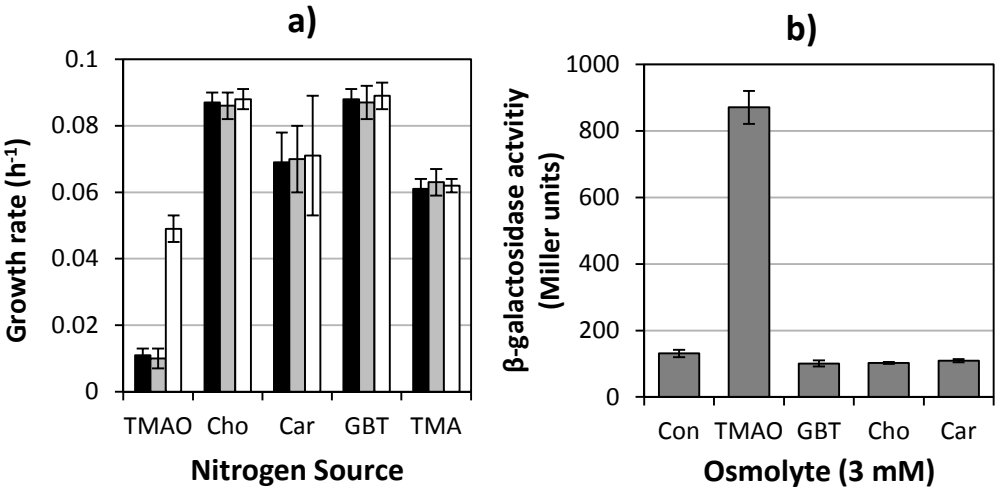

a)
b)
c)
d)
e)
Biomass (OD_{540})
TMA/TMAO concentration (µM)
Time (hours)
10
1
0.1
0.01
700
600
500
400
300
200
100
0
0
20
40
50
60
80
100


a)
SAR11 Clade
Deltaproteobacteria
Gammaproteobacteria
Marine Roseobacter Clade
99
81
98
97
100
Methylopila sp. M107
Methylocella silvestris BL2
Synechococcus sp. PCC 7335
Non-marine Tdm
0.05
b)
DMA concentrtation (µM)
1000
800
600
400
200
0
NO IPTG
0.2mM IPTG
DSS-3
HIMB59
pET28a
tdm homolog


α
δ
γ
Marine Roseobacter clade
SAR11
Ruegeria pomeroyi DSS-3
Roseovarius sp. TM1035
Roseovarius sp. 217
Roseobacter sp. Azwk-3b
Ruegeria sp. Tw15
Roseobacter sp. SK209-2-6
Roseibium sp. TrichSDK4
Roseovarius nubinhibens ISM
Rhodobacterales sp. HTCC2255
Candidatus Pelagibacter ubique HTCC1002
Candidatus Pelagibacter ubique HTCC1062
Candidatus Pelagibacter sp. HIMB5
Candidatus Pelagibacter sp. HIMB59
Deltaproteobacterium SCGC AAA003-F15
Uncultured marine bacterium HF700
Gammaproteobacterium SCGC AAA076-D13
250kb
137 kb
261 kb
11 kb
5 kb
TMAO transporter (tmoXWV)
TMA monooxygenase (tmm)
TMAO demethylase (tdm)
TMAO-sensing two-component regulator (torRTS)
NMG synthase (mgsABC)
GMA synthetase (gmaS)


Cluster F III
BetX, CaiX, ChoX
TmoX
MRC
δ/γ
SAR11
0.1
Cluster F I, II, IV


a)
b)
c)
Biomass (OD540)
TMA/TMAO concentration (µM)
Time (hours)
Time (hours)
Time (hours)
10
1
0.1
0.01
600
500
400
300
200
100
0
0
50
100


a)
0.1
0.08
0.06
0.04
0.02
0
Growth rate (h-1)
TMAO
Cho
Car
GBT
TMA
Nitrogen Source
b)
1000
800
600
400
200
0
β-galactosidase actvitiy (Miller units)
Con
TMAO
GBT
Cho
Car
Osmolyte (3 mM)

Supplementary information

SI Materials and Methods

Construction of marker-exchange mutants in *Ruegeria pomeroyi* DSS-3

To construct a *tdm* mutant, we amplified a region towards the 5'end (with *Pst*I/*Eco*RI and *Xba*I sites engineered in) and a region towards the 3' end (with a *Hin*dIII and *Xba*I engineered in) of the target gene (SPO1562). The two regions were subcloned, along with a gentamycin gene cassette, amplified from p34S-Gm (1), and inserted at an *Xba*I site between the two regions, into the cloning vector, pGEM-T (Promega). The entire construct was ligated into the suicide vector pK18mobsacB at sites *Pst*I and *Hin*dIII. The plasmid was transformed into *E. coli* S17.1 *via* electroporation and mobilised into *R. pomeroyi via* conjugation, using ½ YTSS as the medium (DSMZ). Transconjugants were selected for on the sea salts minimal medium as described in (13) with gentamicin (10 µg ml^{-1}) and monomethylamine (MMA) (3 mM) as a sole nitrogen source. Double crossover mutants were selected by their sensitivity to kanamycin and homologous recombination was confirmed by PCR and DNA sequencing.

To construct a *tmm* mutant, a 770 bp region of SPO1551 (*tmm*) was amplified by PCR (primers used are listed in supplementary Table S4) and subsequently cloned into pK18mobsacB *via Xba*I and *Hin*dIII sites. A gentamycin gene cassette was released from plasmid p34S-Gm using *Sal*I, which was then inserted into pK18mobsacB. The resulting plasmid was transformed into *E. coli* S17.1 *via* electroporation and mobilised into *R. pomeroyi* DSS-3 *via* conjugation. Double crossover mutants were selected as described above and confirmed by PCR and DNA sequencing.

Complementation of the *tdm* mutant with the native *tdm* of *R. pomeroyi* DSS-3 and the *tdm* of *Pelagibacter* sp. strain HIMB59

The promoter of the *tdm* in *R. pomeroyi* was amplified with an *Xba*I and an *Nde*I site engineered at 5' and 3' end, respectively, and subcloned into pGEM-T vector. The construct was released from pGEM-T and inserted into the pET28a containing the *tdm* from either *R. pomeroyi* or strain HIMB59. The promoter and gene were released and inserted into the broad-host range plasmid pBBR1MCS-km at sites *Xba*I/*Eco*RI for the native *tdm* of *R. pomeroyi* and sites *Xba*I/*Bam*HI for *tdm* of strain HIMB59. The plasmid was transformed *via* electroporation into *E. coli* S17.1 and then mobilised into the *tdm* mutant *via* conjugation. Transconjugants were selected for as described above, but replacing the gentamicin with kanamycin (80 μg ml^{-1}).

Complementation of *tmoX* mutant in *R. pomeroyi* with its native *tmoX*

As *tmoX* appeared to be toxic to the *E. coli* JM109 competent cells, we amplified the promoter and *tmoX* gene separately. We amplified the promoter (250 bp upstream region) of *tmoX* (engineering the sites *Hin*dIII and *Bam*HI at the 5' and 3' end, respectively) and sub-cloned it into pGEM-T. We then amplified the *tmoX* gene (engineering the sites *Bam*HI and *Xba*I at the 5' and 3' end, respectively) and subcloned into pGEM-T. The *tmoX* gene was released from pGEM-T and ligated into the plasmid pBBR1MSC-km using the engineered restriction sites. The promoter was subsequently ligated in at sites *Hin*dIII and *Bam*HI. The ligation mixture was desalted using the NucleoSpin Gel and PCR clean up kit (Macherey-Nagel) and transformed into *R. pomeroyi via* electroporation. The settings used were 2.5 kV/mm, 200 amp resistance and 25 Ω capacitance. The time constant varied between 3.9-4.5 ms.

Electrocompetent cells were prepared by modifying the protocol in (2). Briefly, *R. pomeroyi* was grown in a minimal medium with glucose (10 mM) as the carbon source and ammonium (16 mM) as the nitrogen source. 50 ml of cells were incubated at 30 ^{O}C until the cultures

reached an OD_{540} ~0.4. Cells were washed 4 times with ice cold sterile 10% (v/v) glycerol to remove salts and then resuspended in a final volume of 2 ml and 50 µl aliquot were rapidly frozen in dry ice/ethanol. Aliquots were stored at -80 ^{O}C until use.

Overexpression of Tdm in *Escherichia coli.*

The *tdm* gene from *Ruegeria pomeroyi* DSS-3 was amplified by PCR (primers used are listed in Table S4) and subcloned into the pGEM-T vector (Promega). The *tdm* gene was then excised using the *Nde*I/*Eco*RI sites and ligated into the expression vector pET28a (Merck Biosciences). The *tdm* gene from *Pelagibacteraceae* strain HIMB59 was chemically synthesized with *E. coli* codon usage (GenScript Corporation). The synthesized gene was inserted into the expression vector pET28a using the *Nde*I/*Bam*HI sites. The resulting plasmids were then transformed into the expression host *Escherichia coli* BLR(DE3) pLysS (Merck Biosciences). To overexpress Tdm, *E. coli* cells were grown at 37 °C to an OD_{600} of 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.2 mM and TMAO was added to a final concentration of 1 mM. Cultures were then incubated at 25^{o}C for 20 hours prior to assaying supernatant for DMA production from TMAO on a cation-exchange ion chromatograph equipped with a Metrosep C4/250 mm separation column and a conductivity detector (Metrohm).

References

1. Dennis JJ & Zylstra GJ (1998) Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacterial genomes. *Applied and Environmental Microbiology* 64(7):2710-2715.
2. Sebastian M & Ammerman JW (2009) The alkaline phosphatase PhoX is more widely distributed in marine bacteria than the classical PhoA. *ISME J* 3(5):563-572.
3. González JM, *et al.* (2003) *Silicibacter pomeroyi* sp. nov. and *Roseovarius nubinhibens* sp. nov., dimethylsulfoniopropionate-demethylating bacteria from marine environments. *International Journal of Systematic and Evolutionary Microbiology* 53(5):1261-1269.
4. Schäfer A, *et al.* (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145(1):69-73.
5. Kovach ME, *et al.* (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166(1):175-176.
6. Todd JD, Kirkwood M, Newton-Payne S, & Johnston AWB (2012) DddW, a third DMSP lyase in a model *Roseobacter* marine bacterium, *Ruegeria pomeroyi* DSS-3. *ISME J* 6(1):223-226.

Supplementary Figure Legends

Supplementary Figure S1. Phylogenetic distribution of the *tdm* gene among marine bacteria. Nodes at some of the major branched with high bootstrap values (500 replicates) are indicated.

Supplementary Figure S2. Abundance of Tmm and Tdm at sites throughout the Global Ocean Survey. Protein abundances are given per 100,000 reads. Protein sizes were normalised against RecA.

Supplementary Figure S3. Neighbour joining phylogenetic tree showing the distribution of *tmoX* among marine bacteria. High bootstrap values (500 replicates) at some of the major nodes are shown. IMG gene numbers are given in brackets. Where no IMG gene number possible, NCBI accession number was used.

Supplementary Figure S4. Growth of the TMAO transporter mutant of *Ruegeria pomeroyi* (*ΔtmoXW::Gm*) on TMA (grey circles) and TMAO (white circles) as a sole nitrogen source. TMA (grey diamonds) and TMAO (white diamonds) concentrations were quantified throughout the growth. Cultures were grown in triplicate and error bars denote standard deviation.

Supplementary Figure S5.

a) Growth of *Δtmm::Gm* mutant on methylated amines as a sole N source.

b) Wild type *Ruegeria pomeroyi* DSS-3 containing the *tmoX-lacZ* fusion plasmid, pBIL101 were grown in a defined medium in the presence of TMA (1 mM) or TMAO (1 mM) with

succinate (8 mM) as the carbon source, or in the presence of TMA (0.5 mM) or TMAO (0.5 mM) with additional ammonium (4 mM) (TMA+N; TMAO+N) and succinate (8 mM). β-galactosidase activities were assayed in triplicate and error bars denote standard deviations.

c) The *R. pomeroyi* DSS-3 mutant (*Δtmm::Gm*) containing pBIL101 was grown for 16 hours in the presence of additional TMA (0.5 mM) or TMAO (0.5 mM) and β-galactosidase activities were assayed in triplicate. Growth was performed in a defined medium with succinate (8 mM) as the carbon source and ammonium as the nitrogen source (4 mM). Error bars denote standard deviations.

Supplementary Table S1. Growth of *R. pomeroyi* genotypes on methylated amines

Strain	MMA	DMA	TMAO	TMA	NH_4^+
Wild type	+	+	+	+	+
Δtmm::Gm	+	+	+	-	+
Δtdm::Gm	+	+	-	-	+
ΔtmoX::Gm	+	+	-	+	+
ΔtmoXW::Gm	+	+	-	+	+

Supplementary Table S2. Distribution of *tmm* and *tdm* among isolates from the marine *Roseobacter* clade and growth of marine *Roseobacter* clade isolates on TMA and TMAO

Strain	*tmm*	*tdm*	TMA	TMAO
Citreicella sp. E45	+	+	+	+
Citreicella sp. 357	+	+	NT	NT
Dinoroseobacter shibae DFL12	-	-	-	-
Jannashia sp. CCS1	-	-	NT	NT
Labrenzia aggregata IAM 12614	-	-	NT	NT
Labrenzia alexandrii DFL-11	-	-	NT	NT
Loktanella sp. CCS2	-	-	NT	NT
Loktanella vestfoldensis SKA53	-	-	NT	NT
Maritimibacter alkaliphilus HTCC2654	-	-	NT	NT
Nautella italic R11	-	-	NT	NT
Oceanibulbus indolifex HEL-45	-	-	NT	NT
Oceanicola batsensis HTCC2597	-	-	-	-
Oceanicola granulosus HTCC2516	-	-	NT	NT
Octadecabacter antarcticus 238	+	+	NT	NT
Octadecabacter antarcticus 307	-	-	NT	NT
Pelagibaca bermudensis HTCC2601	+	+	NT	NT
Phaeobacter gallaeciensis 2.10	-	-	NT	NT
Phaeobacter gallaeciensis BS107	-	-	-	-
Roseibium sp. TrichSKD4	-	+	NT	NT
Roseobacter denitrificans OCh 114	+	+	+	+
Roseobacter litoralis Och 149	+	+	+	+
Roseobacter sp. AzwK-3b	+	+	NT	NT
Roseobacter sp. MED193	-	-	-	-
Roseobacter sp. SK209-2-6	-	+	-	+
Roseovarius nubinhibens ISM	+	+	+	+
Roseovarius sp. 217	+	+	+	+
Roseovarius sp. TM1035	+	+	+	+

Ruegeria lacuscaerulensis ITI-1157	-	-	NT	NT
Ruegeria pomeroyi DSS-3	+	+	+	+
Ruegeria sp. TW15	-	+	NT	NT
Ruegeria sp. TM1040	-	-	NT	NT
Ruegeria sp. TrichCH4B	-	-	NT	NT
Sagittula stellata E-37	-	-	-	-
Sulfitobacter sp. EE-36	-	-	-	-
Sulfitobacter sp. GAI101	-	-	NT	NT
Sulfitobacter sp. NAS-14.1	-	-	NT	NT
Thalassobium sp. R2A62	+	+	NT	NT
Rhodobacterales bacterium HTCC2083	+	+	NT	NT
Rhodobacterales bacterium HTCC2150	+	+	NT	NT
Rhodobacterales bacterium Y4I	+	+	NT	NT
Rhodobacteraceae bacterium KLH11	-	-	NT	NT
Rhodobacterales sp. HTCC2255	+	+	NT	NT

NT: not tested.

Supplementary table S3. List of bacterial strains and plasmids used in this study

Plasmids/ strains	**Description/use**	**Reference**
Escherichia coli BLR(DE3) pLysS	Host for heterologous protein expression	Promega
E. coli S17.1	Electrocompetent cells used for conjugation	Lab collection
E. coli JM109	Routine host for cloning	Promega
Ruegeria pomeroyi DSS-3	Wild type	(3)
Ruegeria pomeroyi DSS-3 *Δtmm::Gm*	Wild type with disrupted *tmm*	This study
Ruegeria pomeroyi DSS-3 *Δtdm::Gm*	Wild type with disrupted *tdm*	This study
Ruegeria pomeroyi DSS-3 *Δ*+DSS-3	*tdm* mutant complemented with pBIL001	This study
Ruegeria pomeroyi DSS-3 *Δ*+HIMB59	*tdm* mutant complemented with pBIL002	This study
Ruegeria pomeroyi DSS-3 *ΔtmoXW::Gm*	Wild type with disrupted *tmoXW*	This study
Ruegeria pomeroyi DSS-3 *ΔtmoX::Gm*	Wild type with disrupted *tmoX*	This study
Ruegeria pomeroyi DSS-3 *Δ+tmoX*	*tmoX* mutant complemented with pBIL101	This study
p34S-Gm	Source of a gentamycin gene cassette	(1)
pK18mobsacB	Suicide vector for *R. pomeroyi*, Kan^R	(4)
pBBR1MCS-km	Broad host-range plasmid (Kan^R)	(5)
pBIO1878	Spc^R derivative of pMP220 with *lacZ* reporter gene	(6)
pBIL001	SPO1562 (*tdm*) and its promoter cloned into pBBR1MCS-km	This study
pBIL002	*tdm* of *Pelagibacter* strain HIMB59 and the promoter of SPO1562 cloned into pBBR1MCS-km	This study

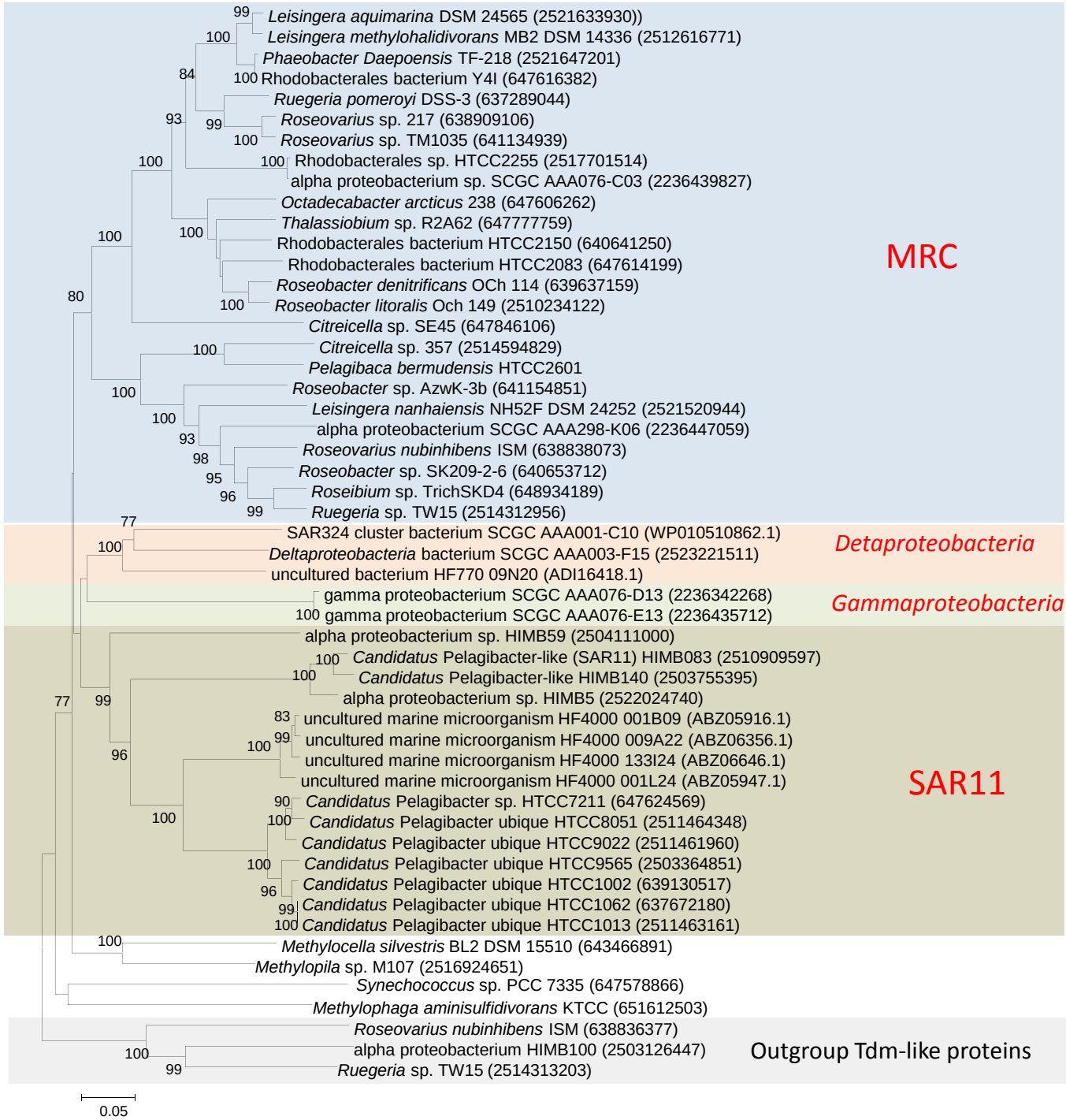
pBIL101	SPO1548 (*tmoX*) and its promoter cloned into pBBR1MCS-km	This study
pKIL101	Internal fragment of SPO1562 (*tdm*) and the Gm^R cassette cloned into pK18mobsacB	This study
pKIL201	Internal fragment of SPO1548 (*tmoX*) and SPO1549 (*tmoXV*) and the Gm^R cassette cloned into pK18mobsacB	This study
pKIL202	Internal fragment of SPO1548 (*tmoX*) and the Gm^R cassette cloned into pK18mobsacB	This study
pBIOIL101	SPO1548 (*tmoX*) promoter cloned into pBIO1878	This Study

Supplementary Table S4. List of all PCR primers used in this study

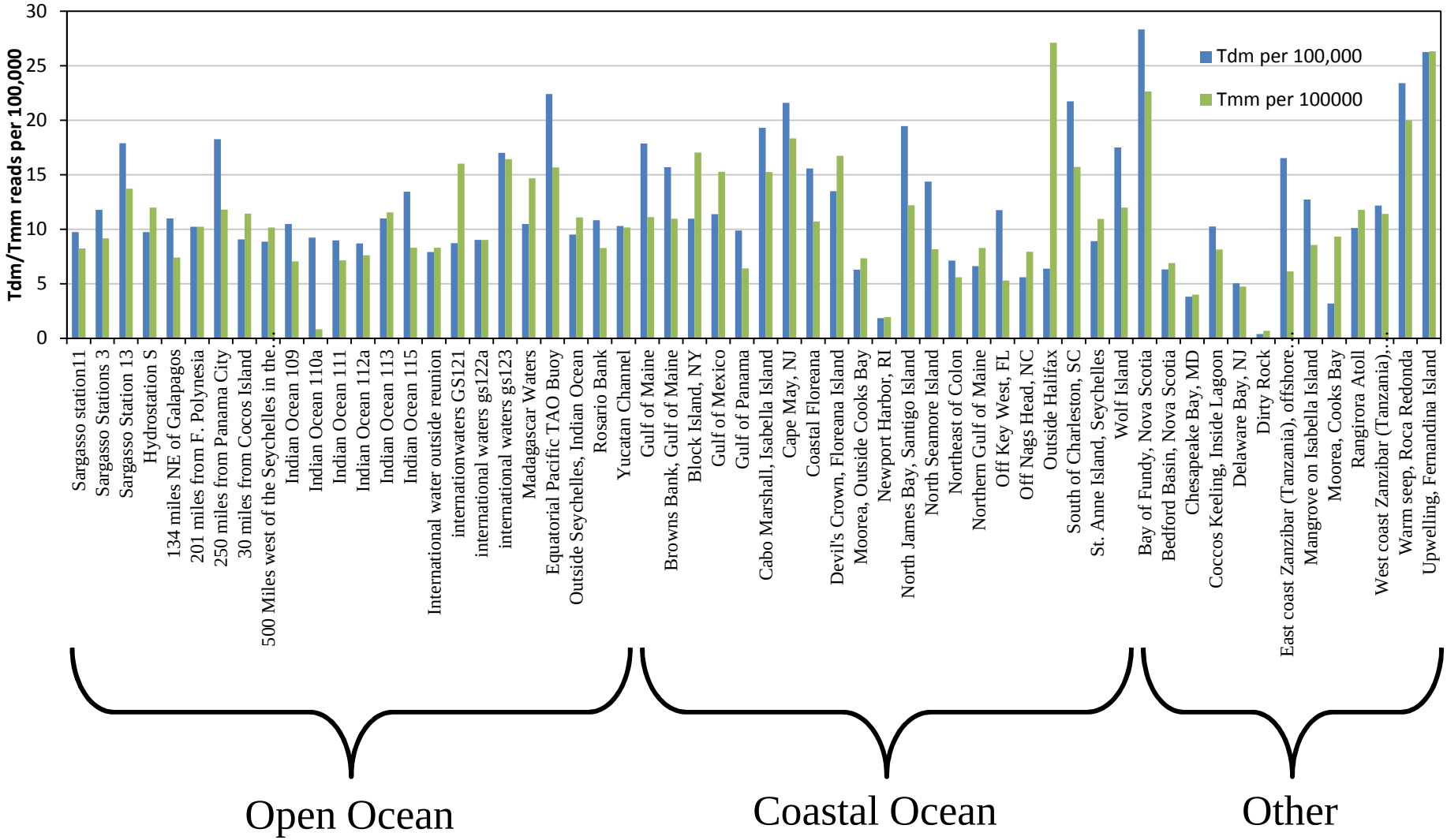
Primer	Sequence	Used for
Tdm_AF1_EcoRI	ATCAGGAATTCACCGTGTGAGATCGTCTGTG	Cloning region A of SPO1562 (*tdm*)
Tdm_AR1_XbaI	AATGCTCTAGAACACTGGAAATCGGTGCATT	Cloning region A of SPO1562 (*tdm*)
Tdm_BF1_XbaI	AATGCTCTAGAGTCTATACCGCCATGTGCT	Cloning region B of SPO1562 (*tdm*)
Tdm_BR1_PstI	CAATGCTGCAGTAGCCGGCAAAGATCAACC	Cloning region B of SPO1562 (*tdm*)
Tdm_CONF_F1	GAACGGAACGCTATGTGGTT	Confirmation of *Δtdm:Gm*
Tdm_CONF_F2	TCTCCATCCGGTCGTAAAAG	Confirmation of *Δtdm:Gm*
Tmm_F_XbaI	GTTACGTCTAGACGCTGGATCGACTACAATGA	Cloning of SPO1551 (*tmm*)
Tmm_R_HindIII	GTTACGAAGCTTGCCACCAGTTCCTTGACGTA	Cloning of SPO1551 (*tmm*)
Tmm_CONF	TCTGGAATTCGCCGACTATT	Confirmation of *Δtmm:Gm*
Tmm_CONR	AGATACGCCTCCATGCTGTC	Confirmation of *Δtmm:Gm*
TmoX_AF_HindIII	CAATAAGCTTTCGCTCTGCTTTGACATGAG	Cloning region A of SPO1548
TmoX_AR_XbaI	CAATTCTAGAAAAGGCCCCTTCCCACAC	Cloning region A of SPO1548
TmoX_BF_XbaI	CAATTCTAGAACTTTGCCGAAGCGGTCT	Cloning region B of SPO1548
TmoX_BR_PstI	CAATCTGCAGGCGCGAATATCGTCGAAC	Cloning region B of SPO1548
TmoX_CONF_F1	ATCTGCGCGAGGAACATAAC	Confirmation of *ΔtmoX:Gm*
TmoX_CONF_R1	AAAGGACTGGAACACCATGC	Confirmation of *ΔtmoX:Gm*
TmoXW_AF_HindIII	CAATAAGCTTGAAATCGCTGCAAATGATCC	Cloning region A of SPO1548
TmoXW_AR_XbaI	CAATTCTAGAACCGGACCATCCAGATAGC	Cloning region A of SPO1548
TmoXW_BF_XbaI	CAATTCTAGAGGGCGCGAGGATTATTTC	Cloning region B of SPO1549
TmoXW_BR_PstI	CAATCTGCAGGCTTGCCTTCAACAGGATGT	Cloning region B of SPO1549
TmoXW_CONF_F1	CCGTTCGATTTGGTCGTATT	Confirmation of *ΔtmoXW:Gm*
TmoXW_CONF_R1	ATGTCCCATTGTCCGATCAT	Confirmation of *ΔtmoXW:Gm*
Tdm_DSS-3_F1_NdeI	CAATCATATGATGCTGGATACCAAATATCCCGAGAT	Cloning SPO1562 (*tdm*)
Tdm_DSS-3_R1_EcoRI	CAATGAATTCTCAAGAGCGGGGTCTGGTTTTCTGCG	Cloning SPO1562 (*tdm*)
Tdm_prom_F1_XbaI	CAATCATATGGTTGCCACTCCGGTCATTTG	Cloning the promoter of SPO1562
Tdm_prom_R1_NdeI	CAATTCTAGAAACCCCAGCCCGGTCGCCAG	Cloning the promoter of SPO1562
TmoX_Prom_F_KpnI	CAATGGTCCAATTCAAAATCAACGCGCAAT	Cloning the TmoXWV promoter *lac*
TmoX_Prom_R_PstI	CAATCTGCAGGCCGCCGAACCTGGAGAGAGTG	Cloning the TmoXWV promoter for
TmoX_F1_BamHI	CAGAGGATCCGTGCGATTGTTTCGAGAAATCGC	Cloning SPO1548 (*tmoX*)

TmoX_R1_XbaI	CAATTCTAGAGATTAGCCGTCCAGCCAGGGGCG	Cloning SPO1548 (*tmoX*)
TmoX_Prom_F2_HindIII	CAATAAGCTTATTCAAAATCAACGCGCAAT	Cloning the promoter of SPO1548
TmoX_Prom_R2_BamHI	CAATGGATCCGCCGCCGAACCTGGAGAGAGTG	Cloning the promoter of SPO1548

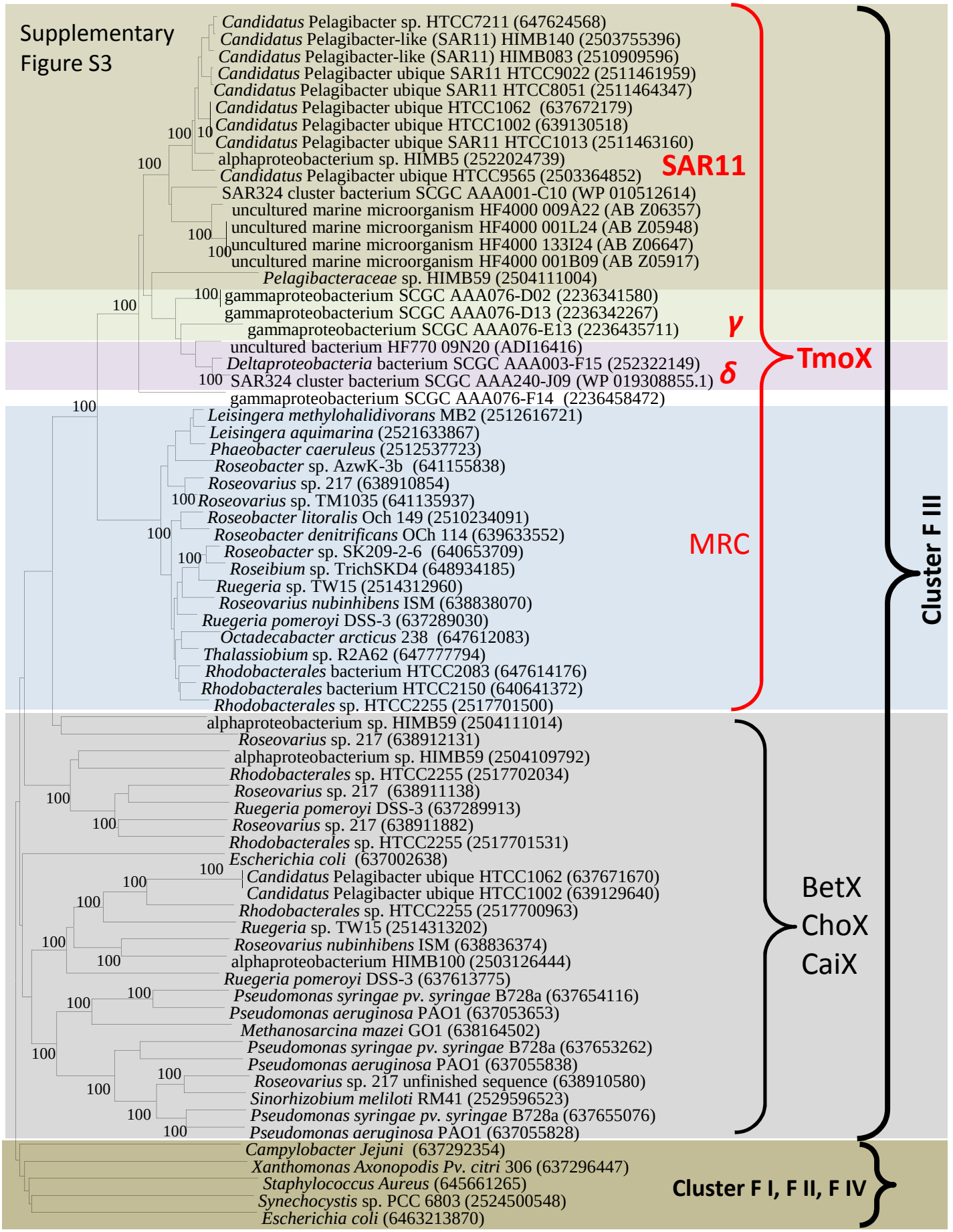
Supplementary Figure S1



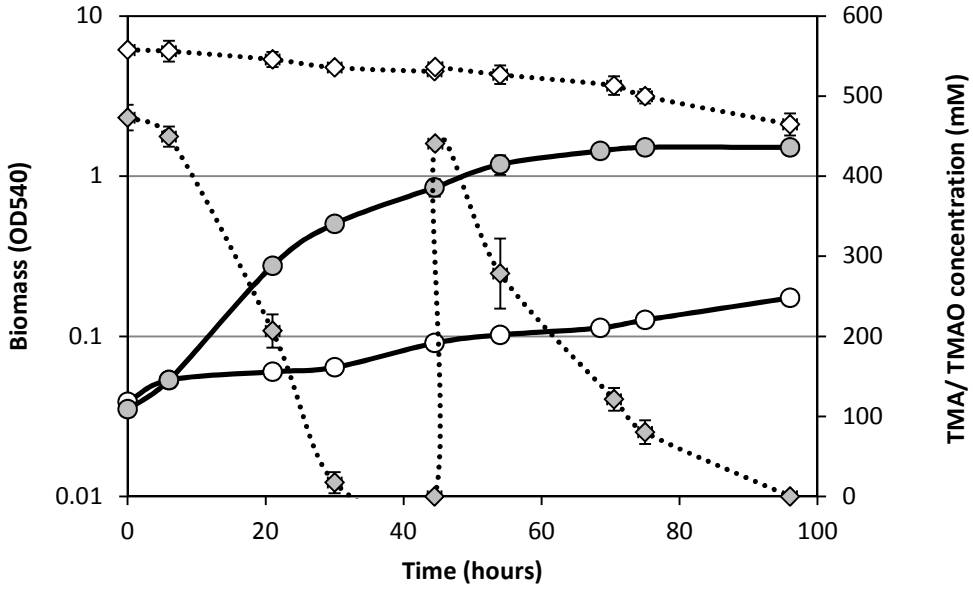
Supplementary Figure S2

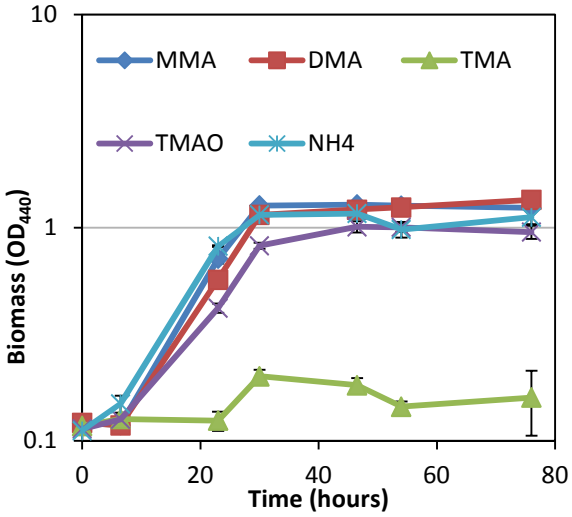


Supplementary Figure S3

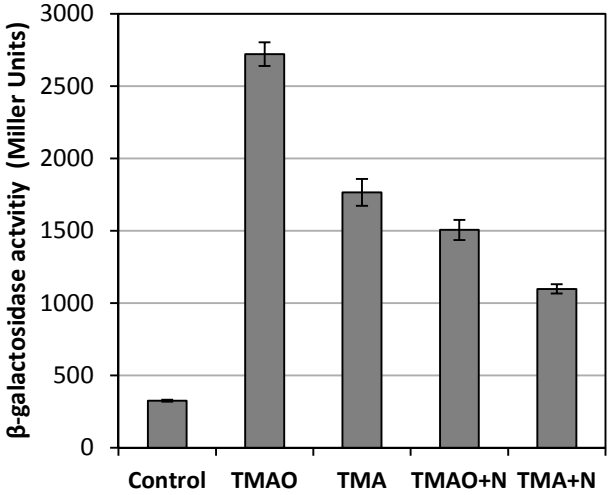


Supplementary Figure S4

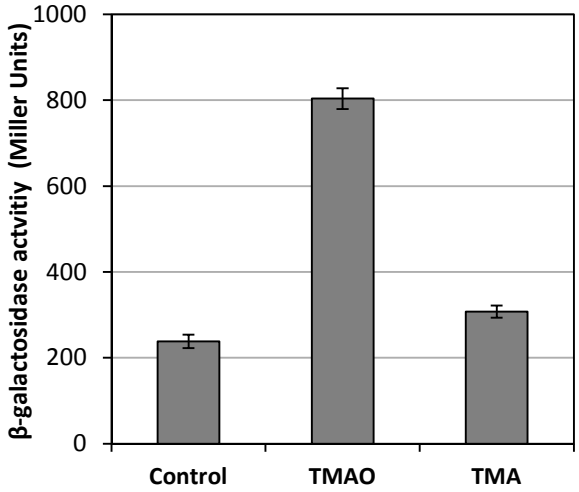



10
1
0.1
Biomass (OD440)
MMA
DMA
TMA
TMAO
NH4
0
20
40
60
80
Time (hours)

a)


3000
2500
2000
1500
1000
500
0
β-galactosidase actvitiy (Miller Units)
Control
TMAO
TMA
TMAO+N
TMA+N

b)


1000
800
600
400
200
0
β-galactosidase actvitiy (Miller Units)
Control
TMAO
TMA

c)